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13. ABSTRACT (Maximum 200 words)

We have answered fundamental questions regarding the mechanisms of attachment and the nature of the biocomposite adhesives utilized by the marine biofouling diatom *Achnanthes longipes* and the freshwater *Cymbella cistula*. During the course of this grant we have: 1) delineated the sequence of events involved in attachment of the organisms to a variety of surfaces; 2) discovered that initial adhesion is mediated by different methods/polymers on hydrophilic surfaces vs. those more hydrophobic and that bacterial "preconditioning" has variable effects on adhesion; 3) developed methodology for mass culture of fouling diatoms and isolation of adhesive components; 4) characterized the "proteoglycan" bioadhesives using monosaccharide and methylation analysis, NMR and other analytical techniques; 5) localized specific carbohydrate moieties of the adhesive with lectins and produced monoclonal antibodies against the adhesives and applied them as probes of structure/function of the adhesives; 6) determined that adhesion is disrupted at several levels by dichlorobenzonitrile (DCB) and related specific inhibitors of plant extracellular polysaccharide synthesis; 7) ascertained that DCB and other potential anti-fouling chemicals act intracellularly on an 18 KD membrane associated protein and that DCB doped polyimide surfaces do not inhibit adhesion; 8) discovered that adhesive structures are not assembled in the presence of high concentrations of iodide and that bromide is a limiting requirement for adhesion; 9) created an expression library to screen for a 50 kD polypeptide from the adhesive and a peroxidase involved in crosslinking the adhesive.

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GRANT TITLE: Diatom Attachment at Aquatic Interfaces: Molecular Interactions, Mechanisms, and Physiology of Adhesion

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OBJECTIVES: 1) Determine the structure and mechanism of biogenesis of the specific components of diatom extracellular secretions that mediate attachment during biofouling; 2) investigate external factors that influence biogenesis of these polymers and adhesion, and; 3) delineate initial events in the diatom adhesion sequence.

APPROACH: We have characterized the diatom adhesion process and bioadhesive properties by: 1) examining the initial sequence of events involved in attachment by video microscopy and other techniques; 2) determining the effects of growth conditions on adhesive production using gradients of numerous parameters, including nutrients, nutrient ratios, heavy metals, temperature, pH, light quality and innoculum cell density; 3) elucidating the influence of bacteria, bacterial secretions and substratum type on adhesion; 4) mass culturing large quantities of biofouling diatoms and developing methods to isolate the bioadhesives; 5) characterizing the adhesives utilizing monosaccharide and methylation analysis, NMR and other analytical techniques; 6) creating a cDNA expression library to screen for polypeptide components of adhesives; 7) developing/utilizing specific molecular probes directed against the adhesives including monoclonal and polyclonal antibodies and various lectins to characterize/localize various components of the adhesive biocomposite; 8) examining the effects of inhibitors/factors that disrupt the process of attachment including specific metabolic inhibitors such as 2,6-dichlorobenzonitrile (DCB) and related compounds, and bromide deprivation and iodide toxicity, and; 9) application of high resolution cryo-scanning electron microscopy (SEM) and freeze-fracture of cryo-preserved specimens to reveal details of structures at the adhesion interface.

ACCOMPLISHMENTS: We have answered fundamental questions regarding the mechanisms of attachment and the nature of the biocomposite adhesives utilized by the marine fouling diatom *Achnanthes longipes* and the freshwater *Cymbella cistula*.

Active attachment of *A. longipes* requires *de novo* secretion of extracellular polymers, whereas the passive mode appears to involve mainly hydrophobic interactions with existing polymers (Wang, 1995; Wang *et al.* 1997). Cells and isolated EPS quickly attach to hydrophobic surfaces, such as polystyrene or silanized glass, in random orientations relative to the substrate, while attachment is much slower with raphe mediated active attachment on hydrophilic surfaces. Video microscopy revealed a consistent sequence of colonization of glass culture vessels by *A. longipes*. Secretions from the raphed valve allow motility for 6-8 h following inoculation. After 6 h, cells cease movement and sequentially produce a basal pad, a ring-like collar

adjacent to the frustule and a cylindrical shaft (diameter 6-7 um) at a rate of 6.5 um/hr. that elevates the cell from the substratum. Stalked cells undergo cell division forming cell filaments composed of up to 100 cells. Proximal cells disengage from cell filaments and initiate the adhesion cycle once again by exhibiting raphe mediated motility. Electron microscopy of adhesive stalks shows multi-component structures with a complex circular multi-layer organization throughout the shaft (Wang, et al. 1997). Cryo-preservation coupled with FESEM (field-emission scanning electron microscopy) has revealed details of structures at the adhesion interface that correlate very well with light microscopical observations (Wang, *et al.* [in preparation]).

Attachment of *A. longipes* was shown to depend on the surface properties of the substratum and the way in which these properties are altered by the presence of bacteria and their exopolymers (Gawne, *et al.* [submitted]). Fewer diatom cells attached to agar or annealed 3140 RTV than to glass or polystyrene (a hydrophobic surface). A bacterial film promoted diatom attachment to glass, had no effect on 3140 RTV, and inhibited attachment to polystyrene. All four bacterial species inhibited diatom attachment to polystyrene, however the attachment of *A. longipes* to surfaces coated with bacterial exopolymers was similar to those with living bacteria.

Achnanthes exhibits an absolute requirement for bromide for stalk production and substratum attachment, while elevated iodide concentrations in the growth medium inhibit insoluble extracellular polymer formation and adhesion (Johnson, 1995; Johnson, *et al.*, 1995). Alterations in bromide and iodide levels resulted in varying extracellular adhesive morphologies (including pads, stalk-pads and none). If sulfate is replaced with methionine, ability to form stalks is lost even in the presence of bromide, suggesting that sulfate is required for cross-linking of stalk polymers. A bromine dependent peroxidase mediated assembly of extracellular polymers may explain the observed effects of bromine and iodide on adhesive morphology and synthesis.

DCB and related herbicides reversibly disrupt the attachment sequence in *A. longipes* without significantly affecting other cellular functions (Wang, 1995; Wang et al., 1997). Raphe mediated attachment and motility and stalk synthesis are inhibited by DCB, which is directly related to the ability of cells to permanently attach to submerged substrates. We have synthesized a fluorescent analog of DCB, 5-methylamino-naphthalene-1-sulfonyl-(3-cyano-2,4-dichloro)aniline (DCBF), that reversibly inhibits adhesive synthesis and interacts specifically with an 18 kD polypeptide isolated from a membrane fraction of *Achnanthes* (Lu, *et al.* [submitted]). We also synthesized a polyimide surface doped with DCB functional groups. Diatoms colonized and completed their adhesion sequence equally well on this DCB-polyimide film as on glass, providing an indication that DCB inhibition of adhesive production is effected intracellularly.

Extracellular adhesives from the diatoms *A.longipes, Amphora coffeaeformis, C. cistula* and *C. mexicana* were characterized by GC-MS, ^{13}C-NMR, lectin-FITC localization and cytochemical staining (Wustman, et al., 1997). Polysaccharide is the major component of adhesives formed during cell motility, synthesis of a basal pad, and/or production of a highly organized shaft. *A. coffeaeformis* and *Achnanthes longipes* adhesives are predominately galactosyl, fucosyl and glucuronosyl

residues, while those for *C. cistula* and *C. mexicana* consist of 75-87% galactosyl and xylosyl residues. Protein components of mechanically isolated adhesive structures of *A. longipes* have been separated with PAGE with the major band from adhesive protein at approximately 50 kD. We have created a cDNA expression library from *Achnanthes*. We are screening this library for a putative haloperoxidase and the 50 kD adhesive protein.

We have produced monoclonal antibodies against extracellular adhesives of *Achnanthes*. Our work yielded four monoclonals and five additional extended colonies (Wustman, *et al.* 1998). These antibodies have a high affinity for solublized extracellular matrix and each shows a unique adhesive labeling pattern. Fractionation of solubilized adhesives with gel filtration and ion exchange chromatography indicates molecular weight and charge heterogeneity and monoclonal antibodies showed differential affinity for column isolated polymers.

The compilation of references generated during writing of our comprehensive review of diatom extracellular polymeric substances (Hoagland *et al.*, 1993) has been updated to the present and now represents a significant database (+5000 entries). In 1995 we organized a forum for distinguished scientists from around the world to present recent results of their work on algal attachment mechanisms in a symposium held in conjunction with the annual meeting of the Phycological Society of America in August.

SIGNIFICANCE: Identification of bromide as an absolute requirement for the formation of the principal attachment organ provides a unique and powerful tool for initiating synchronous stalk production on a large scale and facilitating further research. Our findings regarding the relative influences of bacteria, their exopolymers, and the fundamental character of the substratum material illustrate the complexity of the relationship between fouled surfaces and attaching organisms, and the importance of polymer bridging in the biofouling process. Several monoclonal antibodies directed against the adhesive of *Achnanthes* provide powerful tools for isolation, localization and analysis of adhesive components. Detailed chemical characterization of antibody epitopes provides requisite information to determine the nature of the interactions responsible for assembly of the adhesive. DCB inhibition of diatom adhesion and our discovery of an 18 kD DCB-binding protein in *Achnanthes* parallels results from D. Delmer who reported an 18 kD DCB-binding protein during selective inhibition of cellulose synthesis in cotton. Observation of hydrated specimens using cryo-scanning electron microscopy provides high resolution images of the attachment site and, when combined with localization of colloidal gold conjugated antibodies/lectins, allows for determination of the importance of various epitopes in the adhesion process.

Our current model of the adhesion process in *A. longipes* is as follows: Initial transient attachment is mediated by existing extracellular polymers. The force required to remove the biofoulers at this point is minimal. Synthesis of adhesives occurs shortly following first contact at specialized areas in the cell wall. These adhesives attach the foulers firmly to a wide variety of surfaces and are primarily sulfated fucoglucuronogalactans crosslinked by O-glycosidic sugar-protein linkages into large (>20,000,000 MW) proteoglycan assemblages. The adhesives are synthesized and extruded from the cell wall prior to

extracellular assembly into a sticky biocomposite. The first synthesized adhesives are involved in cell motility and are highly sulfated. After motility ceases, several lines of evidence point to participation of phenolic crosslinking of extruded polymers to create more robust adhesion structures.

PUBLICATIONS, ABSTRACTS & THESES FROM ONR SUPPORT (1993-97):

Wang, Y, Chen, Y., Wustman, B.A. and Gretz, M.R. Immunogold localization of diatom adhesives in high-pressure frozen cells and high resolution SEM images. [In preparation for Protoplasma].

Lu, J., Xia, M. and Gretz, M.R. Synthesis of a fluorescent analog of the herbicide 2,6-dichlorobenzonitrile, an inhibitor of cell wall polysaccharide synthesis. [Submitted to Anal. Biochem.]

Gawne, B. Wang, Y., Hoagland, K.D. and Gretz, M.R. Role of bacteria and bacterial exopolymer in the attachment of Achnanthes longipes (Bacillariophyceae). [Submitted to Biofouling].

Wustman, B.A., Wetherbee, R., Lind, J. and Gretz, M.R. 1998. Extracellular matrix assembly in diatoms (Bacillariophyceae) III. Organization of fucoglucuronogalactans within the adhesive stalks of Achnanthes longipes. Plant Physiol. [in press].

Wang, Y., Lu, J., Mollet, J-C., Gretz, M.R. and Hoagland, K.D. 1997. Extracellular matrix assembly in diatoms (Bacillariophyceae). II. 2,6-dichloro-benzonitrile inhibition of motility and stalk production in the marine diatom *Achnanthes longipes*. Plant Physiol. 113:1071-1080.

Wustman, B.A., Gretz, M.R. and Hoagland, K.D. 1997. Extracellular matrix assembly in diatoms (Bacillariophyceae). I. A model of adhesives based on chemical characterization and localization of polysaccharides from the marine diatom *Achnanthes longipes* and other diatoms. Plant Physiol. 113:1059-1069.

Kinslow, C. 1997. Molecular biology of Achnanthes longipes (Bacillariophyceae). M. Sc. thesis, Michigan Technological U., pp. 60.

Wustman, B.A., Wang, Y., Lu, Jingjie, Gretz, M.R., Wetherbee, R. and Hoagland, K.D. 1996. Extra-cellular bioadhesives from the marine fouling diatom *Achnanthes*. (abst.) Keystone Symp., Tamarron, CO.

Johnson, L.M., Hoagland, K.D. and Gretz, M.R. 1995. Effects of bromide and iodide on stalk secretion in the biofouling diatom *Achnanthes longipes*. J. Phycol. 31:401-412.

Johnson, L.M. 1995. Physiology of *Achnanthes longipes* Ag.: Nutritional requirements for the secretion of extracellular polymeric substances. Ph.D. thesis, U. Nebraska, pp. 173.

Gretz, M.R. 1995. Diatom adhesion mechanisms - relationships between raphe associated motility and stalk mediated attachment in *Achnanthes* and *Cymbella*. J. Phycol. 31(suppl.) (abst.)

Wang, Y. 1995. A study of adhesion mechanisms of the marine biofouling alga, *Achananthes longipes* (Bacillariophyceae). M. Sc. thesis, Michigan Technological U., pp. 73.

Wustman, B.A., Gretz, M.R. and Hotchkiss, A.T. 1994. Uronic acid containing diatom mucilages: evaluation of three methods for characterization of acidic polysaccharides. (abst.) ASLO/PSA, Miami.

Gretz, M.R., Hoagland, K.D. and DeNicola, D.M. 1993. Diatom adhesion mechanisms: characterization of stalks of *Achnanthes longipes* and *Cymbella cistula*. J. Phycol. 29(suppl.):(abst.).

Hoagland, K.D., Rosowski, J.R., Gretz, M.R. & Roemer, S.C. 1993. Diatom extracellular polymer substances: function, fine structure, chemistry and physiology. J. Phycol. 29:537-566.